

# Altered metabolism of familial Alzheimer's disease-linked amyloid precursor protein variants in yeast artificial chromosome transgenic mice

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Missense mutations in the  $\beta$ -amyloid precursor protein gene (*APP*) co-segregate with a small subset of autosomal dominant familial Alzheimer's disease (FAD) cases wherein deposition of the 39-43 amino acid  $\beta$ -amyloid ( $A\beta$ ) peptide and neurodegeneration are principal neuropathological hallmarks. To accurately examine the effect of missense mutations on APP metabolism and  $A\beta$  production *in vivo*, we have introduced yeast artificial chromosomes (YACs) containing the entire ~400 kbp human *APP* gene encoding APP harboring either the asparagine for lysine and leucine for methionine FAD substitution at codons 670 and 671 (APP<sub>K670N/M671L</sub>), the isoleucine for valine FAD substitution at codon 717 (APP<sub>V717I</sub>) or a combination of both substitutions into transgenic mice. We demonstrate that, relative to YAC transgenic mice expressing wild-type APP, high levels of  $A\beta$  peptides are detected in the brains of YAC transgenic mice expressing human APP<sub>K670N/M671L</sub> that is associated with a concomitant diminution in the levels of  $\alpha$ -secretase-generated soluble APP derivatives. Moreover, the levels of longer  $A\beta$  peptides (species terminating at amino acids 42/43) are elevated in YAC transgenic mice expressing human APP<sub>V717I</sub>. These mice should prove valuable for detailed analysis of the *in vivo* effects of the *APP*FAD mutations in a variety of tissues and throughout aging and for testing therapeutic agents that specifically alter APP metabolism and  $A\beta$  production.

## INTRODUCTION

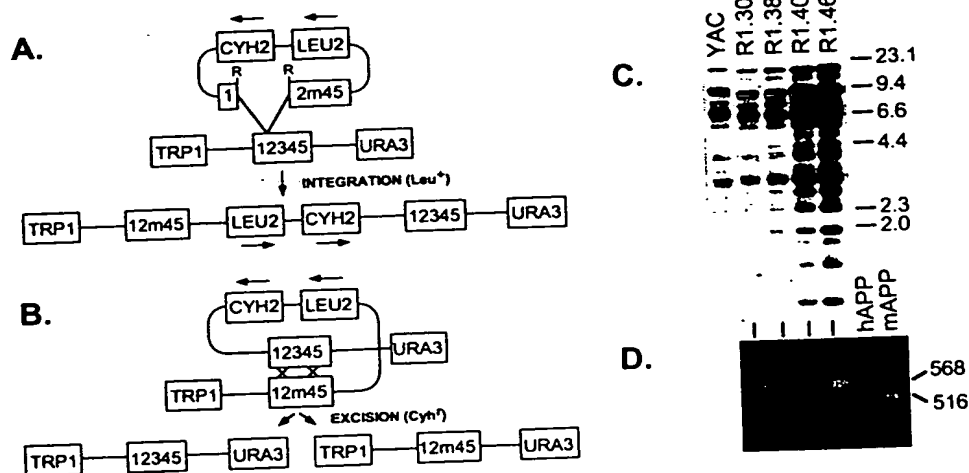
Alzheimer's disease (AD), the most common cause of dementia in the elderly, is a major source of disability and death. Genetic

investigations have identified several etiologies for AD (reviewed in 1), including: a dosage imbalance for chromosome 21, as occurs in Down syndrome (DS); mutations in the  $\beta$ -amyloid precursor protein gene (*APP*) on chromosome 21, the *presenilin 1* gene on chromosome 14 and the *presenilin 2* gene on chromosome 1 in autosomal dominant early onset familial AD (FAD); inheritance of the *apolipoprotein E4* allele on chromosome 19 as a genetic risk factor for both late onset FAD and sporadic AD. A principal cytological hallmark of all AD cases is parenchymal deposits of  $\beta$ -amyloid ( $A\beta$ ), a 39-43 amino acid peptide derived from APP (2).

The *APP* gene encompasses 18 exons and ~400 kbp of DNA that gives rise to at least four tissue-specific alternatively spliced transcripts that encode proteins of 695, 714, 751 and 770 amino acids (3-7). APP, a type I integral membrane glycoprotein of unknown function, matures through the constitutive secretory pathway and is processed by several pathways, including: cleavage by  $\alpha$ -secretase at position 16 of  $A\beta$ , resulting in secretion of the APP ectodomain, thus precluding  $A\beta$  formation; degradation through endosomal-lysosomal pathways; cleavage by  $\beta$ - and  $\gamma$ -secretases at the N- and C-termini of  $A\beta$  respectively, resulting in production and secretion of  $A\beta$  peptides (reviewed in 8). Missense mutations in *APP* occur in a small subset (<5%) of individuals with early onset FAD. In two large related FAD pedigrees in Sweden (9) a double mutation at codons 670 and 671 (of APP-770) results in substitution of lysine for asparagine and methionine for leucine (APP<sub>K670N/M671L</sub>). In ~12 FAD pedigrees (10-16) mutations within the transmembrane domain lead to substitutions of either isoleucine, phenylalanine or glycine for valine at residue 717 of APP-770 (APP<sub>V717I</sub>, APP<sub>V717F</sub> and APP<sub>V717G</sub>).

Studies of transfected cells have provided enormous insights into the mechanism(s) whereby the FAD mutations affect APP processing and  $A\beta$  production. For example, cells expressing APP<sub>K670N/M671L</sub> secrete higher levels of all  $A\beta$ -containing peptides as compared with cells expressing wild-type (wt) APP (17-19); concomitantly,  $\alpha$ -secretase-generated soluble APP

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**Figure 1.** Introduction of mutations into APP YACs and ES cells. (A) A mutation (m) is introduced *in vitro* into a given DNA sequence contained on a YAC (with *TRP1* and *URA3* arms) and cloned into plasmid p680 (30), which contains the yeast *Leu2* and *Cyh2* genes. Cutting the plasmid DNA at a unique site (R) on one side of the mutation as well as introduction of the linear molecule into yeast containing the YAC and selection for *Leu*<sup>+</sup> transformants result in targeted integration of the mutation as well as introduction of the linear molecule into the YAC. (B) Subsequent selection for *Cyh*<sup>r</sup> transformants results in excision of the plasmid and generation of the plasmid to the corresponding genomic sequence on the YAC. (C) High molecular weight DNA from a yeast strain containing the APP YAC and four representative ES transformants (see Table 1) were digested with *HindIII*, transferred to nylon membranes and hybridized with a human *Alu* repetitive element (5). On the right are shown the sizes of molecular weight markers in kbp. (D) Cytoplasmic RNA from the ES transformants was subjected to RT-PCR analysis with appropriate mouse (mAPP) and human (hAPP) plasmid cDNA controls (5). On the right are shown the sizes of the expected human (568 bp) and mouse (516 bp) products.

derivatives are diminished (17). In addition, cells expressing the APP<sub>717</sub> substitutions specifically secrete elevated levels of longer A $\beta$  peptides (20), extending to residues 42 and/or 43 [A $\beta$ 1–42(43)]. Evidence that A $\beta$ 1–42(43) is a highly pathogenic A $\beta$  species derives from biophysical studies showing the highly fibrillogenic nature of the peptide and immunocytochemical studies of brains of AD and DS individuals, demonstrating that A $\beta$ 1–42(43) peptides are deposited relatively early in the disease (21–24).

Numerous standard transgenic mice have been generated that express various wt and mutant APP cDNAs or gene fragments from a variety of promoters (reviewed in 25; see also 26–28). Although these studies have revealed that APP transgenic mice can develop age-related A $\beta$  deposits, they have not specifically and accurately examined the effect of the regulated expression of APP<sub>K670N/M671L</sub> and APP<sub>V717I</sub> on the metabolism of APP and A $\beta$  *in vivo*. In contrast, our efforts were designed to introduce the entire ~400 kbp human APP gene harboring the FAD mutations into the germline of transgenic mice, thus allowing for the proper spatial and temporal expression of mutant APP with appropriate splice donor and acceptor sites needed to generate the entire spectrum of alternatively spliced APP transcripts and encoded proteins. A 650 kbp yeast artificial chromosome (YAC) containing the APP gene was mutagenized using a homologous recombination strategy in yeast, introduced into mouse embryonic stem (ES) cells by lipid-mediated transfection and finally transferred into the mouse germline. In brain and peripheral tissues of these mutant APP YAC transgenic mice high levels of human APP mRNA and protein are expressed in a manner that mirrors the spatial and temporal expression pattern of endogenous mouse APP gene products. Furthermore, elevated levels of A $\beta$  peptides are detected in the brains of YAC transgenic mice expressing human APP<sub>K670N/M671L</sub>, with a concomitant

decrease in levels of soluble  $\alpha$ -secretase-generated APP derivatives as compared with mice expressing similar levels of wt APP. Finally, we demonstrate that elevated levels of longer A $\beta$  peptides are detected in YAC transgenic mice expressing human APP<sub>V717I</sub>.

## RESULTS

### Mutagenesis of APP YACs

The APP<sub>K670N/M671L</sub> and the APP<sub>V717I</sub> FAD mutations were introduced into a YAC containing the entire wt 400 kb human APP gene and ~250 kbp of flanking sequences with multiple neomycin resistance expression cassettes in the YAC vector arm by homologous recombination in yeast (29). Briefly, genomic sequences containing the APP exons (16 and 17) to be mutagenized were subcloned by screening an APP YAC genomic sub-library with exon-specific PCR products. The mutations in exons 16 (APP<sub>K670N/M671L</sub>) and 17 (APP<sub>V717I</sub>) were introduced into the respective genomic fragments using PCR-based mutagenesis strategies and then subcloned into the yeast vector p680. p680 contains the yeast *Leu2* gene, which permits growth in the absence of leucine, and the *Cyh2* gene, which confers sensitivity to cycloheximide (30). Finally, two-step gene replacement was performed in yeast to introduce the mutated APP genomic sequences into the 650 kbp APP YAC (Fig. 1A and B). YACs containing the mutated APP genomic sequences were identified by restriction digestion and sequencing of exon-specific PCR products, while the integrity of the mutant YACs was confirmed by restriction digestion, Southern analysis and pulsed-field gel electrophoresis of yeast genomic DNA (data not shown). Intact YACs were identified containing the APP<sub>K670N/M671L</sub> and APP<sub>V717I</sub> FAD mutations separately or both mutations together.

Table 1. Mutant APP YAC ES clones

Colony <sup>a</sup>	Mutation	Expression <sup>b</sup>		<i>Alu</i> profile <sup>c</sup>	Copy no. <sup>d</sup>	Germline
		5'	3'			
Py8.9 <sup>e</sup>	Wild-type	+	nd	+	1	Yes
J1.2	K670N/M671L + V717I	+	nd	+	4-5	No
J1.6	K670N/M671L	+	nd	+	1	No
J1.22	K670N/M671L	++	+	+	1	No
J1.38	K670N/M671L + V717I	+++	+++	+	5-6	No
J1.46	V717I	+++	++	+	3-4	No
J1.58	K670N/M671L + V717I	+	+/-	+	2	No
J1.80	V717I	+++	++	+	2	No
J1.88	V717I	+	nd	+	nd	Yes
J1.96	K670N/M671L + V717I	++	++	+	1	Yes
R1.17	K670N/M671L + V717I	++	++	+	6-8	Yes
R1.30	V717I	+++	+++	+	2	Yes
R1.38	V717I	++	+	+	6-8	Yes
R1.40	K670N/M671L	++++	++++	+	6-8	Yes
R1.46	K670N/M671L + V717I	++++	+++	+	12-16	No

<sup>a</sup>An additional 21 J1 and eight R1 ES clones contained a majority of the mutant APP YAC, but were not injected into mouse blastocysts.

<sup>b</sup>Relative amounts of APP mRNA as determined by RT-PCR (5).

<sup>c</sup>Intactness of *Alu* repetitive element profile as determined by Southern blot analysis (5).

<sup>d</sup>Copy number determined by Southern blot analysis with an APP exon 7 probe that recognizes both mouse and human APP.

<sup>e</sup>Generated in a previous study (5).

### Introduction of mutated APP YACs into ES cells and mice

The results of our transfection studies with mutant APP YACs are presented in Table 1. Purified and concentrated mutated APP YAC DNA was introduced into both J1 and R1 ES cells by lipid-mediated transfection of suspension cultures generating 83 and 48 G418<sup>r</sup> lines respectively. Twenty one J1 and 13 R1 lines were positive for a majority of the YAC by PCR, including the APP promoter, exon 7, exon 16 and exon 17, and *Alu* repetitive element PCR (data not shown). The copy number and integrity of the APP YACs in the resulting ES lines (Table 1) was determined by extensive restriction analysis and hybridization with APP (data not shown) and human *Alu* probes (Fig. 1C) respectively. Integration of YACs containing mutated APP sequences was confirmed by PCR and restriction analysis. Expression of human APP mRNA was determined by reverse transcription-PCR (RT-PCR) analysis (Fig. 1D) with degenerate primers that span the first six exons of mouse/human APP mRNA (5). Several lines were subsequently introduced into mouse blastocysts and numerous chimeras ranging from 10 to 90% ES cell contribution were generated. To date, five separate lines have transmitted ES DNA through the germline.

### APP expression in mutant YAC transgenic mice

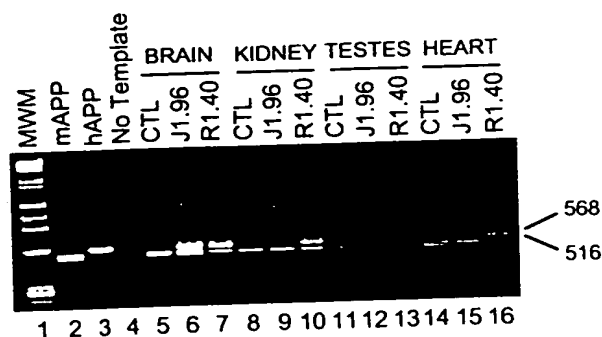
The expression of human APP was examined in several mutant APP YAC transgenic mice, including line J1.96 (which contains both the APP<sub>K670N/M671L</sub> and APP<sub>V717I</sub> mutations) and line R1.40 (which contains only the APP<sub>K670N/M671L</sub> mutation). RT-PCR analysis with degenerate primers that span the first six exons of mouse/human APP mRNA (5) revealed that the J1.96 line, containing a single copy of the mutant APP YAC, expressed human APP mRNA at levels similar to endogenous mouse *App*

mRNA in brain (Fig. 2, lane 6) and peripheral tissues (Fig 2; lanes 9, 12 and 15), while line R1.40, containing ~6-8 copies of the mutant APP YAC, expressed human APP mRNA at levels ~2- to 3-fold above endogenous *App* mRNA in all tissues examined (Fig. 2, lanes 7, 10, 13 and 16). Additional RT-PCR experiments (5) with degenerate primers that flank the most common alternatively spliced APP exons (7 and 8) demonstrated that the levels of transcripts that encode human APP-695, -751 and -770 in selected tissues paralleled the levels of alternatively spliced mouse *App* transcripts in all transgenic lines examined (data not shown).

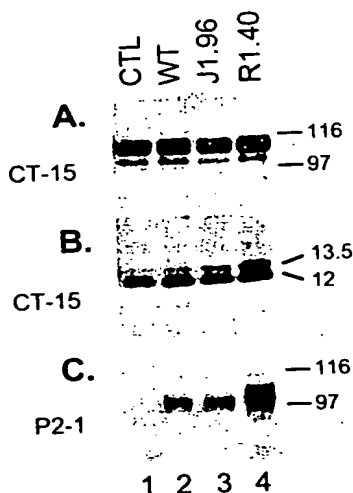
### APP processing in mutant YAC transgenic mice

Expression of human APP-related derivatives in brains of mutant APP YAC transgenic mice was examined by Western blot analysis. We demonstrate that in membrane fractions of brains from line J1.96 accumulation of full-length mutant human APP, recognized by antibody CT-15 specific for the terminal 15 amino acids of mouse and human APP (Fig. 3A, lane 3), is essentially indistinguishable from the level of wt human APP (5) in YAC transgenic line Py8.9 (Fig. 3A, lane 2). However, the levels of an ~13.5 kDa fragment that likely represents a  $\beta$ -secretase generated C-terminal peptide extending from the N-terminus of A $\beta$  to the end of the APP cytoplasmic tail is slightly elevated in membrane fractions of the J1.96 line (Fig. 3B, lane 3) relative to brains of animals expressing wt human APP (Fig. 3B, lane 2), whereas an ~12 kDa fragment that likely represents the  $\alpha$ -secretase-generated C-terminal fragment remains unchanged.

These data are consistent with our earlier demonstration that cultured cells expressing APP<sub>K670N/M671L</sub> accumulate elevated levels of a C-terminal ~13.5 kDa fragment generated following cleavage by  $\beta$ -secretase (17). Reinforcing this view, we also

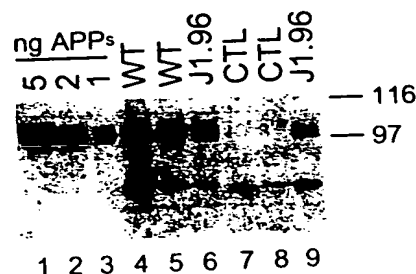


**Figure 2.** Analysis of *APP* mRNA expression in mutant *APP* YAC transgenic mice. Total RNA from the brain (lanes 5–7), kidney (lanes 8–10), testes (lanes 11–13) and heart (lanes 14–16) of a 2-month-old control mouse (CTL), a mouse from line J1.96 (containing both the  $APP_{K670N/M671}$  and  $APP_{V717I}$  mutations) and a mouse from line R1.40 (containing the  $APP_{K670N/M671}$  mutation) was subjected to RT-PCR analysis with appropriate mouse (mAPP), human (hAPP) and no template controls (lanes 1–3). Because the sense primer is  $^{32}P$ -labeled in these experiments, the gel was dried and bands quantitated by phosphorimaging technology. Line J1.96 produces roughly equivalent amounts of mouse and human *APP* mRNA in the brain, while line R1.40 produces ~3-fold higher levels of human *APP* mRNA relative to mouse *App* mRNA. On the right are shown the sizes of the expected human (568 bp) and mouse (516 bp) products.



**Figure 3.** Soluble and cell-associated APP-derived molecules in *APP* YAC transgenic mouse brain. Soluble (S2 fraction) and cell-associated (P2 fraction) protein extracts from the brains of 3-month-old control (CTL; lane 1), wild-type (WT, line Py8.9; lane 2) and mutant (line J1.96, containing both  $APP_{K670N/M671}$  and  $APP_{V717I}$  mutations, and line R1.40, containing the  $APP_{K670N/M671}$  mutation; lanes 3 and 4) *APP* YAC transgenic mice (5) were subjected to Western blot analysis (17,36). P2 extracts subjected to SDS-PAGE (A) and Tris-Tricine-PAGE (B) were visualized with antibody CT-15 (recognizing the C-terminal 15 amino acids of mouse and human APP), while S2 extracts (C) subjected to SDS-PAGE were visualized with antibody p2-1 (recognizing an epitope in the N-terminus of human APP). On the right are shown the approximate sizes in kDa.

document that the amount of the same  $\beta$ -secretase-generated fragment accumulates to very high levels in line R1.40 mice overexpressing high levels of human  $APP_{K670N/M671L}$  (Fig. 3B,



**Figure 4.** Levels of  $\alpha$ -secretase-cleaved APP in mutant *APP* YAC transgenic mouse brain. Soluble (S2 fraction) extracts from the brains of 4-month-old control (CTL; lanes 7 and 8), wild-type (WT, line Py8.9; lanes 4 and 5) and mutant (line J1.96, containing both  $APP_{K670N/M671}$  and  $APP_{V717I}$  mutations; lanes 6 and 9) *APP* YAC transgenic mice (5) and  $\alpha$ -secretase-cleaved APP standards (lanes 1–3) were subjected to Western blot analysis. Parallel blots were visualized with antibody 6E10 (recognizing  $\alpha$ -secretase cleaved human APP; 32) and a horseradish peroxidase tagged antibody (data not shown) against full-length APP. Blots quantified with  $^{125}I$ -labeled protein A revealed an ~40–50% reduction in  $\alpha$ -secretase-cleaved products in mutant line J1.96 relative to wt when normalized to the full-length APP signal. On the right are shown the approximate sizes of the molecular weight markers in kDa.

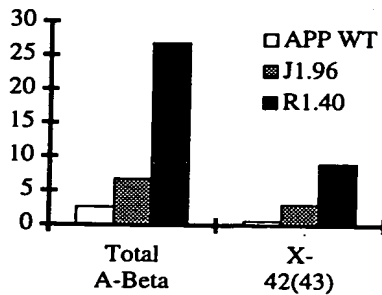
lane 4). Interestingly, the steady-state levels of full-length human APP in line R1.40 (Fig. 3A, lane 4) is ~3-fold higher than endogenous App (Fig. 3A, lane 1), a finding that parallels the RT-PCR studies shown in Figure 2.

Analysis of soluble APP derivatives ( $APP^s$ ) in brains of mutant APP line J1.96 (Fig. 3C, lane 3) and wt APP line Py8.9 (Fig. 3C, lane 2) using an antibody specific for epitopes in the N-terminus of human APP failed to reveal a significant difference in total  $APP^s$  between these lines. However, commensurate with an elevation of  $\beta$ -secretase-generated C-terminal fragments in the brain of line R1.40, we observed elevated levels of accumulated  $APP^s$  (Fig. 3C, lane 4). We extended this analysis to examine the levels of  $\alpha$ -secretase-generated soluble derivatives in lines J1.96 and Py8.9, expressing essentially identical levels of mutant and wt human APP respectively. Western blot analysis of soluble fractions (S2) from brains revealed an ~50% decrease in the steady-state levels of  $\alpha$ -secretase-generated human  $APP^s$  derivatives in the J1.96 line relative to line Py8.9 (Fig. 4), this despite our demonstration that the total levels of  $APP^s$  appeared unchanged between the mutant and wt transgenic lines (Fig. 3C).

Although decreased  $\alpha$ -secretase processing of mutant  $APP_{K670N/M671L}$  appears surprising, these data are fully consistent with our earlier cellular transfection studies which documented that one effect of the  $APP_{K670N/M671L}$  mutation is to enhance cleavage at the  $\beta$ -secretase site in the Golgi compartment (17). As a result, a fraction of APP molecules which would normally be cleared by  $\alpha$ -secretase at the plasma membrane would be effectively disabled by the prior action of  $\beta$ -secretase. Hence, in the medium of cells expressing  $APP_{K670N/M671L}$  the level of  $\alpha$ -secretase derivatives are considerably diminished relative to the medium of cells expressing similar levels of wt APP.

#### A $\beta$ production in mutant *APP* YAC transgenic mice

Levels of A $\beta$  were quantified using sandwich enzyme-linked immunosorbent assays (ELISAs) (20). Analysis of brain homogenates demonstrated an increase both in the amount of



**Figure 5.** Levels of various A $\beta$  peptides in APP YAC transgenic mouse brain. Brain extracts from a 3-month-old control, wild-type (WT, line Py8.9) and mutant (line J1.96, containing both the APP<sub>K670N/M671L</sub> and APP<sub>V717I</sub> mutations, and line R1.40, containing the APP<sub>K670N/M671L</sub> mutation) APP YAC transgenic mice (5) were subjected to a sensitive sandwich ELISA that detects the various A $\beta$  peptides [shown are total A $\beta$  and A $\beta$ X-42(43)]. Levels are expressed as pmol/g relative to control standardized peptides.

total soluble A $\beta$  and the proportion of longer A $\beta$ 1-42(43) in the mutant APP J1.96 line compared with the wt APP line Py8.9 (Fig. 5, A $\beta$ X-42(43) is 40% of total A $\beta$  in line J1.96 compared with 20% for line Py8.9). Moreover, a dramatic increase in both total A $\beta$  and A $\beta$ 1-42(43) species was observed in line R1.40. Our preliminary studies of [<sup>35</sup>S]methionine-labeled primary cortical neuronal cultures and immunoprecipitation of A $\beta$  from conditioned medium revealed a substantial increase in A $\beta$  in the mutant APP line J1.96 relative to wt APP line Py8.9 or control cultures (data not shown). These *in vivo* results, similar to those obtained in cell culture experiments and human plasma, suggest that the effect of the APP<sub>K670N/M671L</sub> FAD mutation is to enhance  $\beta$ -secretase cleavage, resulting in increased production and secretion of A $\beta$ , while the APP<sub>V717I</sub> mutation appears to influence processing at the  $\gamma$ -secretase site in a manner that elevates the levels of secreted A $\beta$ 1-42(43).

## DISCUSSION

A $\beta$ , the principal component of parenchymal amyloid deposits in AD, is derived from the integral membrane glycoprotein APP. Several mutations have been identified in the APP gene that co-segregate with affected individuals in FAD pedigrees (reviewed in 1). Although multiple APP transgenic mice have been generated in attempts to develop animal models of AD (reviewed in 25; see also 26-28), the molecular mechanisms involved in altered APP metabolism and A $\beta$  deposition in individuals with FAD-linked mutations in APP have not been fully clarified. The present report provides insight into the effects of the APP<sub>K670N/M671L</sub> and APP<sub>V717I</sub> FAD mutations on APP metabolism and A $\beta$  production in an *in vivo* setting that mimics the conditions occurring in individuals with the FAD mutations. We have generated lines of transgenic mice that contain the entire human APP gene carried on a 650 kbp YAC with the APP<sub>K670N/M671L</sub> mutation, the APP<sub>V717I</sub> mutation or both mutations together. In these mice mutant human APP transcripts and protein(s) are expressed in spatial and temporal patterns similar to the endogenous mouse products in both brain and peripheral tissues. Western blot analysis and sensitive ELISAs of whole brain homogenates demonstrated that in mice expressing APP<sub>K670N/M671L</sub> cell-associated C-terminal APP fragments

containing the A $\beta$  domain and A $\beta$  peptides were elevated concomitant with a reduction in the levels of release of  $\alpha$ -secretase-cleaved APP products. Mice expressing APP<sub>V717I</sub>, on the other hand, accumulated elevated levels of longer A $\beta$ 1-42(43) species in the brain.

One additional consideration in these YAC transgenic experiments is the presence of an additional gene(s) carried on the APP YAC that might alter the effect of the FAD mutations when expressed at dosage imbalance. Initial experiments with exon trapping from the 650 kbp APP YAC revealed the presence of five additional exons that do not encode for APP (J.J. Yeh, B.T. Lamb and J.D. Gearhart, unpublished observations). While four of these exons did not reveal substantial identity with sequences in the database, one trapped exon of 103 nt revealed complete identity with bp 167-269 (exon 2) and amino acid residues 1-25 of the human transcription factor GABPA (E4TF1-60) gene (GenBank accession no. D13318), consistent with the localization of this gene to chromosome 21q21.2 (31,32). This transcription factor contains an ets-related DNA binding domain which binds to DNA at CGGAAA/G sequences and forms heterodimers with other polypeptides (33). Although it remains unclear whether the entire GABPA gene is present on the 650 kbp APP YAC, the biochemical abnormalities observed in the mutant APP YAC transgenic mice likely reflect a property unique to the mutant APP polypeptide and not dosage imbalance for GABPA, as we have generated both wt and mutant APP YAC transgenic mice that express APP at a similar level (i.e. wt line Py8.9 and mutant line J1.96).

In summary, our results suggest that *in vivo* both FAD mutations influence APP processing in a manner leading to increased levels of the highly amyloidogenic peptide A $\beta$ 1-42(43); mice expressing APP<sub>K670N/M671L</sub> accumulate increased levels of all A $\beta$  peptides, while mice expressing APP<sub>V717I</sub> selectively accumulate elevated levels of A $\beta$ 1-42(43). In view of *in vitro* aggregation and immunocytochemical studies which have suggested that A $\beta$ 1-42(43) is highly amyloidogenic and deposited early in the disease process, we are enthusiastic that the APP YAC mice described herein will develop amyloid deposits and associated lesions, as has been described in mice expressing other FAD-linked APP transgenes (26-28). In addition, these YAC transgenic mice will allow an accurate examination of the effects of the FAD mutations on APP processing in a variety of cell types (neurons, astrocytes, glia, blood vessels, etc.) as well as on alterations in neuronal physiology and behavior throughout aging. Finally, our results suggest that the YAC transgenic strategy will allow the stringent testing of the roles of the early onset *presenilin* FAD mutations in amyloidogenesis and examine the potential interactive effects of the different early onset FAD gene mutations.

## MATERIALS AND METHODS

### YAC manipulations

A human APP genomic sub-library was constructed by pulsed-field gel electrophoresis fractionation and isolation of the 650 kbp APP YAC, followed by digestion with *SacI* and ligation into *SacI*-digested  $\lambda$  phage ZAPII (Stratagene). Exon 16 and 17 APP genomic clones were isolated by screening the sub-library with a 229 bp exon 16 (9) and a 319 bp exon 17 product (13), yielding 4.2 and 3.5 kbp genomic clones respectively. Clones

were converted to plasmids and restriction maps generated. A 1.8 kbp *HindIII* fragment containing exon 16 and a 3 kbp *EcoRI* fragment containing exon 17 were subcloned into Bluescript (Stratagene). The APP<sub>K670N/M671</sub> and APP<sub>V717I</sub> mutations were introduced into exon 16 and 17 genomic subclones respectively by PCR mutagenesis. Primary PCR was performed with the internal mutagenic primers 5'-GAGATCTCTGAAGTGAATC-TGGATGCAGAATTCCGAC-3' and its complement for the APP<sub>K670N/M671</sub> mutation and 5'-GGACAGTGATCATCATCA-CCTTG-3' and its complement for the APP<sub>V717I</sub> mutation, along with external vector primers. Secondary PCR was performed with the external primers alone, products subcloned into the yeast recombination plasmid p680 (provided by P.Hieter, Johns Hopkins Medical Institutions) and the presence of the mutations confirmed by DNA sequencing. An *AccI* site upstream of the exon 17 genomic clone was converted to a *StuI* site by the addition of linkers.

Plasmids containing the mutagenized sequences were linearized with *StuI*, which cuts upstream of both exon 16 and 17, and introduced into the wt APP YAC by two-step gene replacement in yeast as described (30). The presence of the mutations was determined by restriction digestion of exon-specific PCR products and confirmed by DNA sequencing and Southern blot analysis. Mutated YAC DNA was purified essentially as described (5) with a second dimension electrophoresis step in 4% Nu-Sieve low melting point agarose (FMC) to concentrate the YAC DNA (34).

### Cell culture and blastocyst injections

J1 (kindly provided by R.Jaenisch, The Whitehead Institute) and R1 (kindly provided by J.Rossant, University of Toronto) ES cells were maintained by standard techniques, grown on mitotically inactivated neo<sup>r</sup> primary embryonic fibroblasts in the presence of leukemia inhibitory factor. For transfection, 1 ml isolated YAC DNA was gently mixed with 50 µg lipofectin (BRL) in polystyrene tubes and allowed to complex for 45 min at room temperature. Lipid-DNA complexes were added to 5 × 10<sup>6</sup> ES cells, resuspended in 9 ml OptiMEM (BRL), placed on 10 cm bacterial dishes, incubated for 4 h at 37°C and then plated onto 10 cm tissue culture dishes with feeders. G418 (250 µg/ml) selection was initiated the following day and continued for 11 days. Injection of dissociated ES cells into C57BL/6J blastocysts and matings of the resultant chimeras were carried out essentially as described (35). Genomic DNA was isolated from ES cells and mouse tails and analyzed by PCR or by Southern blot with a variety of probes as previously described (5).

### RNA and protein analysis

RNA was isolated from ES cells and mouse tissues and RT-PCR was performed for the first six exons or across the alternatively spliced exons of mouse/human APP as described previously (5). Protein extracts and immunoblots were prepared as described (17,36). Sandwich ELISAs specific for Aβ<sub>1-40</sub> versus Aβ<sub>1-42(43)</sub> were performed as described (20).

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### ABBREVIATIONS

Aβ, β-amyloid; Aβ<sub>1-42(43)</sub>, Aβ extending to residue 42 or 43; AD, Alzheimer's disease; APP, amyloid precursor protein; APP<sub>s</sub>, soluble APP derivatives; APP<sub>K670N/M671L</sub>, APP with asparagine for lysine and leucine for methionine substitution at codons 670/671; APP<sub>V717I</sub>, APP with isoleucine for valine substitution at codon 717; ES, embryonic stem; FAD, familial Alzheimer's disease; wt, wild-type; YAC, yeast artificial chromosome.

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## NOTE ADDED IN PROOF

Recently, the entire human *APP* gene was sequenced (37) and found to consist of 286 722 bp, considerably smaller than previous size estimates (5). Analysis of the resulting genomic sequence should provide considerable information with regards to additional expressed sequences and/or genes surrounding the *APP* locus.